Full Length Research Paper

Antioxidant activity of a standardized extract of *Byrsonima japurensis* A. Juss. (Malpighiaceae) stem bark

Fernanda Guilhon-Simplicio[1]*, Tatiane Pereira De Souza[1], Alexandre Antônio Alonso[2], Patrícia Danielle Oliveira de Almeida[1], Pierre Alexandre dos Santos[1], Débora Teixeira Ohana[1], Emerson Silva Lima[1] and Maria de Meneses Pereira[1]

[1]Faculdade de Ciências Farmacêuticas, Universidade Federal do Amazonas, Rua Alexandre Amorim, 330, Aparecida, 69010-300, Manaus-AM, Brazil.
[2]Departamento de Ciências da Natureza, Universidade Federal do Piauí - Campus Cinobelina Elvas, Rodovia BR 135, Km 3, Planalto Horizonte, 64900-000, Bom Jesus-PI, Brazil

An infusion of 5% *Byrsonima japurensis* stem bark, an Amazonian medicinal plant, presented anti-inflammatory, anti-hyperalgesic, antiplatelet and antiulcer activity in a previous study. In this study, pharmacognostical parameters for this raw material were determined and the technological characterization of the infusion was performed, concurrently with the assessment of their antioxidant potential. The analysis of the raw material and the crude extract was done using pharmacopoeial and non-pharmacopeial methods. The antioxidant potential was evaluated by diphenylpicrylhydrazyl (DPPH•), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS•+) and nitric oxide (NO•) radical scavenger assays. The raw material presented a mean size of particles of 318.30 ± 1.65 µm, ash content of 3.39 ± 0.04 g%, 10.32 ± 0.09 g% loss on drying and 12.23 ± 0.17 g% of matter extractable by water. The extract obtained by infusion of 5% of plant drug is rich in polyphenol compounds (50.69 ± 3.64 mg GAE/g) with appreciable flavonoid content (2.38 ± 1.15 mg quercetin/g). This extract showed expressive antioxidant activity, which was significantly better than ascorbic acid and Trolox in both DPPH and ABTS radical scavengers tests ($p < 0.05$), which is closely related to its previously detected anti-inflammatory activity

Key words: Infusion, nitric oxide, phenol compounds.

INTRODUCTION

The *Byrsonima* genus (Malpighiaceae) has about 150 species with a remarkable neotropical distribution. In various regions of Brazil, several species of this genus are widely used in the treatment of gastrointestinal complications. Several studies showed good pharmacological potential of these species, which presented antioxidant, antimicrobial (various microorganisms) and anti-inflammatory activities (Guilhon-Simplicio and Pereira,

2011).

Byrsonima japurensis A. Juss. (synonyms: *Byrsonima fluminensis* Nied., *Byrsonima inundata* Benth., *B. japurensis* subspecies *fluminensis* (Nied.) Cuatrec., *B. japurensis* subspecies *silvatica* Cuatrec and *Byrsonima uvulifera* Spruce) is an arboreal species endemic to the Amazon region, and typically found in lowland areas (Missouri Botanical Garden, 2011). In the State of

Amazonas (Brazil), where it is popularly known as "saratudo", these species have wide application in folk medicine against several inflammatory conditions, including gastritis and gastric ulcers. For this reason, the stem bark is scraped and dried in the shade, and tea is produced with the fragments by decoction or infusion, consumed one to two times per day until symptoms disappear.

In this study, we performed the pharmacognostical characterization of *B. japurensis* stem bark and analyzed technological parameters of an aqueous extract obtained by an infusion of 5% of this plant material, which showed anti-inflammatory, anti-hyperalgesic, antiplatelet and antiulcer activities in a previous study (Guilhon-Simplicio et al., 2012). Additionally, we analyzed the antioxidant potential of this standardized extract to determine the pharmacological potential of the species.

MATERIALS AND METHODS

Plant

Samples of stem bark of *B. japurensis* A. Juss. (Malpighiaceae) used in this study were collected in the city of Careiro-Castanho, Amazonas, Brazil, on March 2, 2007. The species was identified by José Lima of the Herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA), where a voucher specimen was deposited under number 224415. Another voucher specimen was deposited in the Herbarium of the Universidade Federal do Amazonas (HUAM) under number 7983. The collection of the samples was authorized by Managing Council of the Genetic Patrimony (CGEN), chaired by the Ministry of Environment (Brazil), under registration 034/2008.

Histochemical analysis

The histochemical analysis of the secondary phloem of *B. japurensis* was performed according to the methodology of Johansen (1940), using ferric chloride for the identification of phenolic compounds, Lugol to identify starch, Dragendorff reagent to identify alkaloids and Sudam III to identify steroids.

Preparation and characterization of the raw material

The bark samples of *B. japurensis* were dried at 40°C in an oven with internal movement of air, until they acquired a brittle aspect. After this period, they were scraped to remove their most external layer and subjected to further drying for another 3 days, and then pulverized in a mechanical grinder with a sieve with mesh size of 0.5 mm. This material obtained is named, vegetal raw materials (VRM) in this work. The analysis of the average size of particles, the loss on drying and the ash content of VRM were performed in quadruplicate in accordance with the recommendations of the Brazilian Pharmacopoeia (Brasil, 2010). The extractive content was analyzed in quadruplicate in accordance with Bundesvereinigung Deutscher Apothekerverbände (1986).

Phytochemical screening

The investigation of coumarins, tannins, flavonoids, steroids, triterpenoids and saponins was carried out according to Matos

(1997), in a 70% hydroalcoholic extract prepared with 5% of VRM. The search for cyanogen heterosides was performed as described in Matos (1997) and the search for cardioactive steroids and alkaloids, as described in Costa (2001).

Preparation of aqueous extract

The extract was obtained by infusion with distilled water and 5% of VRM for 15 min. After this, the extract was filtered twice, first using cotton cloth to remove coarse particles, and the second using analytical filter paper in a vacuum filtration system. Loss of solvent was compensated by supplementing the initial volume with distilled water in a volumetric flask after cooling. Part of the resulting solution was used for determining the relative density, pH (Brasil, 2010), and dry matter content (Bundesvereinigung Deutscher Apothekerverbände, 1986). The other portion was for determining total polyphenol and flavonoid content and to use in the tests of antioxidant activity after lyophilization.

Total phenolic compounds content

The total phenolic content of the extract was determined by the Folin-Ciocalteau method with a few modifications (Benoli et al., 2004). The extract and gallic acid were diluted in ethanol at 1 mg/ml and 10 µl of each solution was transferred to a 96-well plate. Then, we added 50 µl de Folin-Ciocalteau solution (1:10 in distilled water) and the plate was incubated at room temperature for 8 min. After this, we added 240 µl of sodium carbonate at 0.4% and incubated the plate for additional 3 min. Absorbance was measured at 620 nm using a multimode detector spectrometer (DTX 800, Beckman Counter). The analysis was performed in triplicate and the total phenolic content was expressed in mg of gallic acid equivalents (GAE)/g of extract.

Total flavonoid content

The total flavonoid content of the extract was determined in accordance with Chang et al. (2002). Initially, the extract in ethanol at 1 mg/ml was diluted, then 30 µl of the solution was transferred and the quercetin solution (diluted with 80% ethanol at 1 mg/ml) to a 96-well plate. After this, 90 µl of 95% ethanol, 6 µl of aluminum chloride at 10%, 6 µl of potassium acetate at 1 M and 168 µl of distilled water was added in each well. The plate was incubated for 30 min at room temperature and the absorbance was measured at 405 nm in a multimode detector spectrometer. The analysis was performed in triplicate and the total phenolic content was expressed in mg of quercetin equivalents/g of extract.

Diphenylpicrylhydrazyl (DPPH) scavenger activity

Different concentrations of aqueous extracts dissolved in 50% ethanol were incubated in darkness with DPPH solution in 50% ethanol in 96-well plates, in triplicate. After 30 min of incubation at room temperature, the absorbance was measured at 517 nm. All specter measurements were performed with a multimode detector spectrometer. Ascorbic acid, quercetin and Trolox were used as positive control.

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS[+]) scavenger activity

Different concentrations of aqueous extracts dissolved in 50% ethanol were incubated in darkness with ABTS[+] solution in 50%

ethanol in a 96-well plate, in triplicate. After 15 min of incubation at room temperature, the absorbance was measured at 714 nm using a multimode detector spectrometer. Ascorbic acid, quercetin and Trolox were used as positive control.

Nitric oxide (NO·) scavenger activity

This assay was performed with modified Griess Ilosvoy reagent (Govindarajan et al., 2003). A mixture containing 2 ml of sodium nitroprusside (10 mM), 0.5 ml of phosphate buffer (10 mM, pH 7.2) and 0.5 ml of the extract or standard solution of ascorbic acid in different concentrations was incubated at 25°C for 150 min. After this, 0.5 ml of the solution was mixed with 1 ml of sulphanilic acid (0.33% in 20% glacial acetic acid). After 5 min, 1 ml of N-(1-naphthyl)ethylenediamine dihydrochloride (0.1% p/v) was added, then allowed to stand at 25°C for 30 min. The absorbance was measured at 540 nm in a multimode detector spectrometer. Ascorbic acid, quercetin and Trolox were used as positive control.

Statistical analyses

The results are presented as mean ± standard deviation. The median inhibitory concentration (IC_{50}) of the antioxidant activity was calculated by linear regression method using Origin® software. The results were compared by paired Student's t-test considering a p value lower than 0.05 as statistically significant.

RESULTS AND DISCUSSION

The determination of physicochemical parameters of the quality control of vegetal raw materials (VRM) is a very important step to ensure the reproducibility of the therapeutic/biological effects of their sub-products, allowing the choice of appropriate methods for its treatment and extraction (Costa et al., 2011).

In histochemical tests performed on the stem bark of *B. japurensis* lipids, starch and phenolic compounds were detected, mainly accumulated in the cells of the ray and axial parenchyma (Figure 1). Alkaloids were not detected in the sample.

These results of phytochemical screening were consistent with the histochemical screening. No nitrogen compounds were detected. This analysis confirmed that phenolic compounds (anthocyanins/anthocyanidins, aurones, chalcones, flavanones and condensed tannins) and steroid compounds (saponins, pentacyclic triterpenes, cardioactive steroids) are present in the vegetal drug.

The raw material showed loss of weight on drying of 10.32 ± 0.09 g% in extreme conditions, which according to Brazilian Pharmacopeia (Brasil, 2010), favors the maintenance of their characteristics during storage for long periods. During this analysis, it was observed that 3 h at 105°C is sufficient for satisfactory drying of this raw material.

Considering that the outer layer was scraped, the ash content of 3.39 ± 0.04 g% is representative of physiologic ashes. It is reported that anthocyanins, present in plant drug, can form colored complexes with different metals (Harborne and Williams, 2000). On the other hand, this parameter should be used to detect adulterations in the pulverized raw material, since Brazilian Pharmacopeia (Brasil, 2010) allows a maximum of 14% for physiologic ash content.

The particle size of sample directly influences the efficiency of an extraction, since it implies the contact area between the raw material and the solvent. The VRM obtained as described in this study presented particle sizes of 318.30 ±1.65 µm, therefore classified as "moderately fine", being adequate to efficient extraction. Additionally, the expressive extractive content by decoction, 12.23 ± 0.17 g%, indicates that hot extractions with water are good extractive methods for this raw material (World Health Organization 1998; Brasil, 2010). On the other hand, the crude extract obtained by infusion of 5% of VRM presented relative density of 1.0024 ± 3.99 × 10^{-5} g/ml, pH of 6.06 ± 0.02 and dry residue content of 10.61 ± 0.12 g%. We detected a total polyphenol content of 50.69 ± 3.64 mg GAE/g and total flavonoid content of 2.38 ± 1.15 mg quercetin/g.

The phenolic compounds constitute one of the most common groups of substances in plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens (Pandey and Rizvi, 2009). This group is commonly divided in phenolic acids, flavonoids and other compounds with more restricted distributions, such as phenolic amines, stilbenes and lignans (Tsao, 2010).

Thus, considering the results of phytochemical and histochemical screening, and giving the results of the quantification of total flavonoids in our sample, it can be inferred that the difference between total phenol compounds content and total flavonoids content are majorly composed by condensed tannins or proanthocyanidins, which are dimers, oligomers or polymers of catechins, a class of flavonoids (Manach et al., 2004) and phenolic acids, which are widely distributed in the vegetal kingdom (Khadem and Marles, 2010). In fact, gallic acid and a few derivates were previously isolated from different parts of the *Byrsonima* species (Guilhon-Simplicio and Pereira, 2011).

The raw material preparation and the choice of the water for obtaining crude extract are important to a successful extraction, with expressive polyphenol content, which present various pharmacological activities (Middleton et al., 2000), such as antioxidant capacity. The results presented by I5 in the antioxidant activity tests are shown in the Table 1.

The expressive antioxidant capacity of I5, which was higher than that of ascorbic acid and Trolox in DPPH and ABTS radical scavengers tests, can be easily explained by the presence of flavonoids in the extract (Pietta, 2000; Rice-Evans et al., 1999; van Acker et al., 1996). The general structure of flavonoids facilitates the donation of electrons and hydrogen to the oxidized species (Es-Safi et al., 2007).

It is known that the neutralization of free radicals limits their capacity to cause damage to tissues (Machlin and
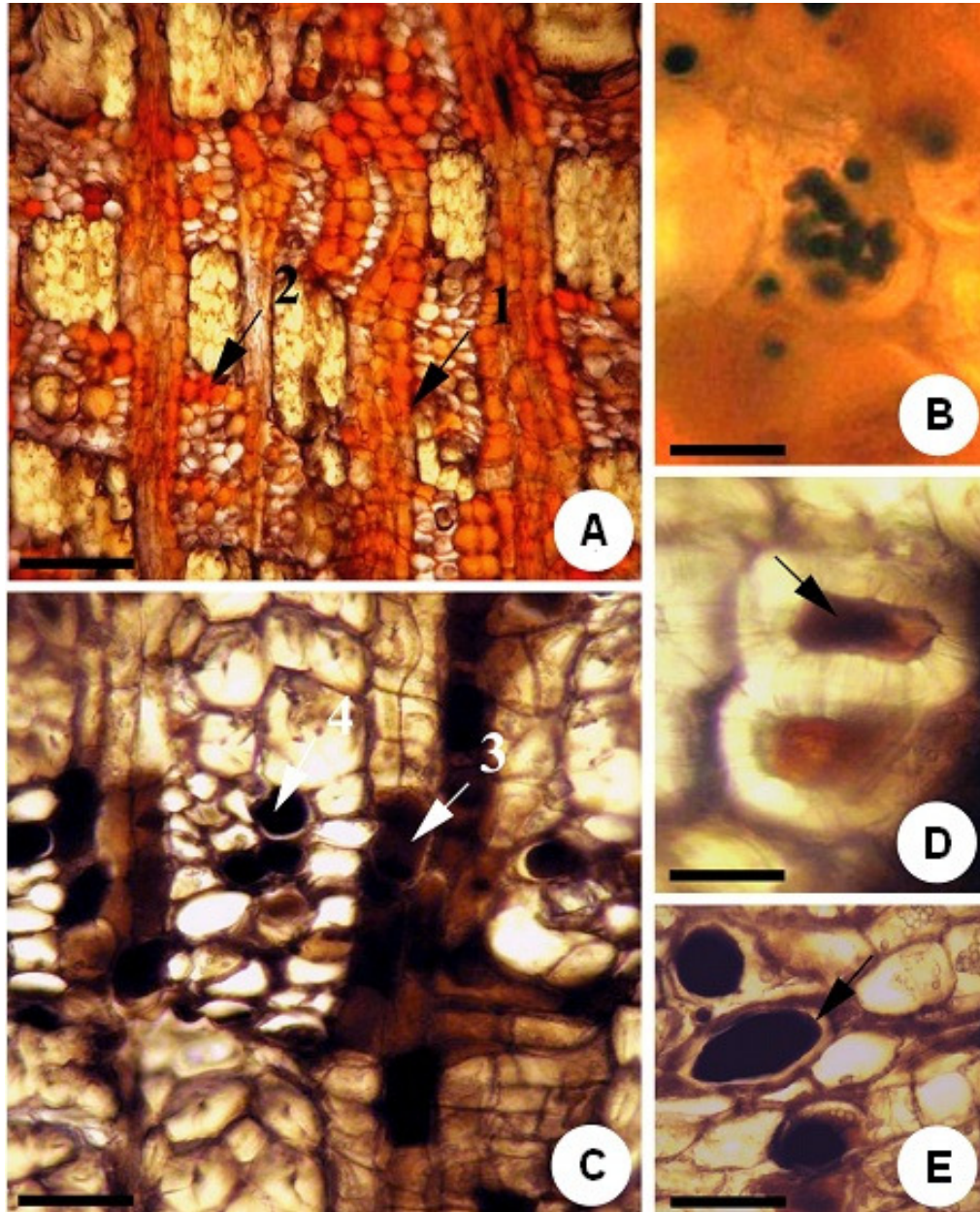
Figure 1. Transverse sections of secondary phloem of *B. japurensis* subjected to histochemical tests. (A) Lipids in radius cells (arrow 1) and in the axial parenchyma cells (arrow 2); (B) Grains of starch in radius cells; (C) Phenolic compounds in radius cells (arrow 3) and in the axial parenchyma cells (arrow 4); (D) Sclereids with phenolic compounds; (E) Detail of parenchyma cells with phenolic compounds.

Table 1. Antioxidant activity of I5.

Drugs tested	DPPH radical	ABTS radical	NO radical
	IC_{50} in X ± s (µg/ml)		
I5	7.1 ± 0.4*	4.2 ± 0.2*	613.2 ± 11.4*
Ascorbic acid	14.7 ± 0.2	10.4 ± 0.8	197.0 ± 1.1
Quercetin	4.1 ± 0.8	2.8 ± 0.4	76.1 ± 1.3
Trolox	11.6 ± 0.5	7.0 ± 0.1	194.4 ± 3.7

*$p < 0.05$ versus all positive control.

Bendich, 1987; Schinella et al., 2002; Wellen and Hotamilisgil, 2005). Furthermore, studies indicate that phospholipases, cyclooxygenases and lipoxygenase inhibition by flavonoids may be due to the neutralization of radicals formed in the active site of these proteins (Cao et al., 1997).

The role of the high antioxidant potential of I5 on the anti-inflammatory activity displayed was previously discussed, which showed that the extract has good superoxide radical scavenger capacity and inhibition of lipid peroxidation, both essential for blocking the tissue damage during the inflammatory response (Guilhon-Simplicio et al., 2012). In turn, the flavilium cation, fundamental core of anthocyanidins and anthocyanins, both present in *B. japurensis*, can neutralize negatively charged radicals, such as superoxide radicals (Nowakowska, 2007).

Here, we emphasized the antioxidant potential of I5 on differently charged radicals; also, we showed that it has the ability to neutralize the nitric oxide radical, which is essential for initiation and propagation of the cascade of lipid peroxidation, one of the main deleterious events associated with inflammatory diseases (Salvemini et al., 2006).

Thus, this paper shows quality control parameters to the stem bark of *B. japurensis*, which are essential for the credibility and reproducibility of the further studies investigating their mechanism of action for pharmacological activities presented previously and others that maybe possibly investigated. According to our results, the extract obtained by infusion of 5% of stem bark is very interesting for further studies, due to their high polyphenol content and expressive antioxidant capacity, which is closely related with their anti-inflammatory activity.

REFERENCES

Benoli M, Verardo V, Marconi E, Caboni MF (2004). Antioxidant phenols in barley (*Hordeum vulgare* L.) flour: comparative spectrophotometric study among extraction methods of free and bound phenolic compounds. J. Agric. Food Chem. 52:5195-5200.

Brasil (2010). Farmacopéia Brasileira, vol. 1, 5nd ed. Brasília: Anvisa, p. 546.

Bundesvereinigung DA (1986). Deutscher Arzneimittel – Codex. Frankfurt: Govi; Stuttgart: Deutscher Apotheker.

Cao G, Sofic E, Prior RL (1997). Antioxidant and pro-oxidant behavior of flavonoids: structure-activity relationships. Free Radical Biol. Med. 22: 749-760.

Chang CC, Yang MH, Wen HM, Chern JC (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal. 10:178-182.

Costa AF (2001). Farmacognosia (3th volume). Lisboa: Calouste Gulbenkian p. 1032.

Costa LM, Santos VA, Ohana DT, Lima ES, Pereira MM, De Souza TP (2011). Technological development of aqueous extract *Calycophyllum spruceanum* (mulateiro) using a factorial design. Rev. Bras. Farmacogn. 21:181-186.

Es-Safi NE, Ghidouche S, Ducrot PH (2007). Flavonoids: hemisynthesis, reactivity, characterization and free radical scavenging activity. Molecules 12:2228-2258.

Guilhon-Simplicio F, Pereira MM (2011). Chemical and pharmacological aspects of *Byrsonima* (Malpighiaceae). Quim. Nova 34:1032-1041.

Guilhon-Simplicio F, Pinheiro CCS, Conrado GG, Barbosa GS, Santos PA, Pereira MM, Lima ES (2012). Anti-inflammatory, anti-hyperalgesic, antiplatelet and antiulcer activities of *Byrsonima japurensis* A. Juss. (Malpighiaceae). J. Ethnopharmacol. 140:282-286.

Govindarajan R, Rastogi S, Vijayakumar M, Shirwaikar A, Rawat AKS, Mehrotra S, Palpu P (2003) studies on the antioxidant activities of *Desmodium gangeticum*. Biol. Pharm. Bull. 26:1424-1427.

Harborne JB, Williams CA (2000). Advances in flavonoid research since 1992. Phytochem. 55:481-504.

Johansen DA (1940). Plant microtechnique. New York: McGraw-Hill. p. 523.

Khadem S, Marles RJ (2010). Monocyclic phenolic acids; hydroxy- and polyhydroxybenzoic acids: occurrence and recent bioactivity studies. Molecules 15:7985-8005

Machlin LJ, Bendich A (1987). Free radical tissue damage: protective role of antioxidant nutrients. Fed. Am. Soc. Exp. Biol. J. 1:441-445.

Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L (2004). Polyphenols: food sources and bioavailability. Am. J. Clin. Nutr. 79: 727-747.

Matos FJA (1997). Introdução à fitoquímica experimental. Fortaleza: Edições UFC p. 136.

Missouri Botanical Garden (2011). *Byrsonima japurensis* A. Juss. http://www.tropicos.org/name/19500195, access in Jun.

Middleton-Jr E, Kandaswami C, Theoharides TC (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52(4):673-751.

Nowakowska Z (2007). A review of anti-infective and anti-inflammatory chalcones. Eur. J. Med. Chem. 42:125-137.

Pandey KB, Rizvi SI (2009). Plant polyphenols as dietary antioxidants in human health and disease. Oxid. Med. Cell. Longev. 5:270-278.

Pietta PG (2000). Flavonoids as antioxidants. J. Nat. Prod. 63:1035-1042.

Rice-Evans CA, Miller NJ, Paganga G (1999). Antioxidant properties of phenolic compounds. Trends Plant Sci. 2:152-159.

Salvemini D, Doyle TM, Cuzzocrea S (2006). Superoxide, peroxynitrite and oxidative/nitrative stress in inflammation. Biochem. Soc. Trans. 34:965-970.

Schinella GR, Tournier HA, Prieto JM, Mordujovich de Buschiazzo P, Ríos JL (2002). Antioxidant activities of anti-inflammatory plant extracts. Life Sci. 70:1023-1033.

Tsao R (2010). Chemistry and biochemistry of dietary polyphenols. Nutrients 2:1231-1246.

van Acker SABE, van den Berg DJ, Tromp MNJL, Griffioen DH, van Bennekom WP, van der Vijgh WJF, Bast A (1996). Structural aspects of antioxidant activity of flavonoids. Free Radical Biol. Med. 20:331-342.

Wellen KE, Hotamilisgil GS (2005). Inflammation, stress and diabetes. J. Clin. Invest. 115:1111-1119.

World Health Organization (1998). Quality control methods for medicinal plant material compounds. Geneva, WHO p 122.